Double immunostaining : WGA labeled neurons and TH immunopositive fibers in the rat mesocortex

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Double Immunostaining: WGA Labeled Neurons and TH Immunopositive Fibers in the Rat Mesocortex

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Abstract: Following a unilateral injection of wheat germ aggulutinin (WGA) into the thalamic MD, retrogradely labeled neurons and TH immunopositive fibers in the mesocortex were examined by double immunostaining. On the ipsilateral side, a number of pyramidal neurons retrogradely labeled with WGA were located in layers V/VI and in layers II/III of the medial mesocortex. Some occasionally appeared in the motor area continuing dorsolaterally but none was found in the somatosensory area. A small aggregation of labeled neurons was noted in the lateral mesocortex, circumscribing the claustrum. The same regions on the contralateral side displayed labeled neurons but the number never exceeded 10% of that counted on the ipsilateral side. The terminals of thalamocortical projections were also visualized as fine grains distributed in the superficial layers on the ipsilateral side. In double immunostained sections, it was clearly demenstreted that WGA labeled pyramidal neurons stained blue were often surrounded by TH varicose fibers stained brown.

Key words: mesocortex, thalamic mediodorsal nucleus, wheat germ aggulutinin, tyrosine hydroxylase, double immunostaining

INTRODUCTION

In the previous experiments, a dense laminar and regional innervation of dopaminergic (DA) terminals was confirmed in prelimbic area (PL), ventral and dorsal anterior cingulate (ACv; ACd) and dorsal agranular insular cortex (AId) by immunohistochemistry for tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH)[1,2,9,10]. These regions correspond to the transitional cortical parts from allocortex to isocortex, that is, mesocortex[15]. Then, the distribution of DA terminals in layers II/III and layers V/VI of the mesocortex has received attention for considering a role of DA fibers in the regions. In order to clarify the mesocortical function, it is necessary to examine DA fiber connections to cortical neurons and to various afferents converging from subcortical areas.

Fiber connections between the cortex and the thalamic or other subcortical nuclei have been examined using various tracers, such as fluorescent dyes, tritiated proline, horseradish peroxidase (HRP) and wheat germ agglutinin

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conjugated with HRP (WGA-HRP)[8,11,14,16,17]. These tracers are useful to label somas retrogradely and terminals anterogradely. In the study combining with light and election microscopic immunohistochemistry, however, enzymatic tracers such as HRP and WGA-HRP may have a problem in causing transneural diffusion or false reaction products. Thus, the present preliminary experiment was performed to assess a single WGA as a tracer for the examination of fiber connections by between the thalamus and the mesocortex prior to applying double immunostaining for WGA and TH at the ultrastractural level.

Materials and Methods

Sixteen male Sprague-Dawley rats weighing 180-250g were anesthetized with nembutal (37.5 mg/Kgbw) and fixed on a stereotaxic apparatus. WGA (Sigma, USA) was injected into the thalamic mediodorsal nucleus (MD) by a Hamilton's syringe (1.5 μl of 2.5% WGA dissolved in saline). The coordinate was determined on the basis of the rat atlas[13]: 2.5 mm posterior to the bregma; 0.5 mm lateral to the midline; 5.5 mm below the skull. After 2-3 days, rats were sacrificed by perfusion fixation. Washing out the blood with saline for 2 min, a fixative (4% paraformaldehyde and 0.25% glutaraldehyde in 0.1M PB at pH 7.4) was perfused through the aorta for 15 min. Excise brains were postfixed overnight and stored in 15% sucrose PB for 1-2 days. Cryostat sections of 16 μm in thickness were processed for immunostaining by avidin-biotin complex (ABC) method. In the double immunostaining, sections were first incubated in anti-WGA antibodies (EY-Lab, USA) diluted to 1 : 100 in PBS for 2 days at 4 ℃, then in biotinylated IgG (Vector, USA) diluted to 1 : 1,000 for 2 hs, and subsequently in ABC solution (Vectastain ABC kit) diluted to 1 : 1,000 for 2 hs. Reaction products were visualized as dark blue deposits in a diaminobenzidine (DAB) solution containing nickel (0.02% DAB, 0.6% nickel ammonium sulfate and 0.06% H_2O_2 in 0.05M Tris buffer at pH 7.6). Thereafter, these sections were restained with TH antibodies (offered by courtesy of Dr. I. Nagatsu) diluted to 1 : 10,000 in PBS for 2 days at 4℃, and in fresh solutions of biotinylated IgG and ABC for 2 hs respectively. Reaction products for TH were visualized as brown deposits in DAB solution (0.02% DAB, 0.06% H_2O_2 in Tris buffer).

Results

Corticothalamic projecting fibers took WGA and retrogradely transported it toward the perikarya in the mesocortex. Engulfed WGA was visualized as granules stained dark blue or diffuse materials in the cytoplasm by WGA immunohistochemistry (Fig. 2C). Most of WGA labeled neurons were large or medium-sized pyramidal cells with an apical dendrite. In the injection site, WGA was restricted within thalamic MD, although the needle track caused bleeding and degeneration.

The number of labeled neurons was tentatively counted in immunostained sections, and the results were illustrated in Fig. 1. In the ipsilateral hemisphere, a large group of WGA labeled neurons was noted in layers V/VI of the medial mesocortex including PL, ACv and ACd (Fig. 2A). The numbe of labeled neurons ranged from 50 to 250 per section (average 160 neurons/section). In layers II/III, WGA labeled neurons were seen in parallel with the pial surface (20-70 neurons/section: average 40). These two groups often extended toward layers III/VI in the medical motor area, where the number of labeled neurons evidently decreased. In the somatosensory area, none of WGA labeled

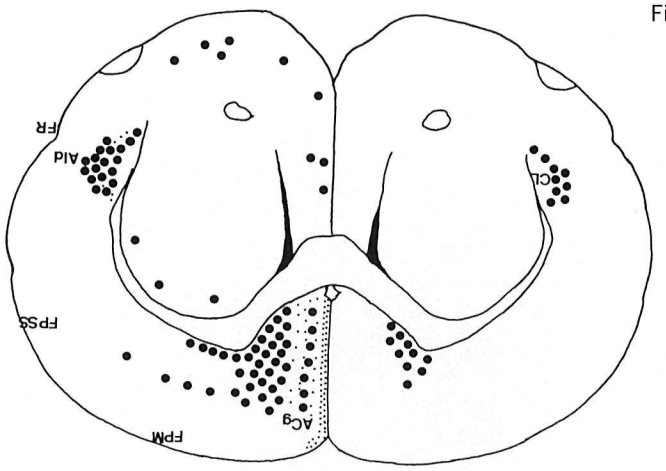

Fig. 1 Schematic presentation of WGA labeled neurons (solid circles) and terminals (small dots). See the text.
ACg: anterior cingulate cortex
AId: dorsal agranular insular cortex
CL: claustrum
FPM: frontoparietal motor cortex
FPSS: frontoparietal somatosensory cortex
FR: rhinal fissure

neurons was found. In the lateral mesocortex, AId, circumscribing the claustrum, an aggregation of WGA labeled neurons were noted (20-130 neurons/section: average 60; Fig. 4A-C). WGA labeled cells in AId seemed to be of pleomorphic or stellate type with no apical dendrite. A few labeled neurons often lay scattered in the claustrum, anterior hippocampal continuation, ventral pallidum and dorsolateral part of the neostriatum (5-20 neurons/section). In the same regions on the contralateral side, some WGA neurons were observed but the number never exceeded 10% of a total of WGA neurons counted on the ipsilateral side.

On the other hand, the neuropil of the ipsilateral mesocortex clearly indicated a dense distribution of grain-like WGA immunodeposits (Fig. 2B). These profiles spread at random but more densely in the superficial layers than in deep layers. They were regarded as the terminals derived from thalamic neurons.

The distribution pattern of TH immunopositive fibers was consistent with that in the previous experiments[1,9,10]. A dense distribution of TH fibers was confirmed in layers V/VI and layers II/III. In double immunostained sections, the perikarya of WGA neurons stained dark blue did clearly contrast with TH fibers stained brown. These TH varicose fibers often surrounded the perikarya of WGA labeled neurons (Fig. 3A-C; Fig. 4B, C).

DISCUSSION

In this preliminary experiment, it was confirmed that WGA is a useful tracer for the immunohistochemical approach to search fiber connections.

Neurons in the cortex are generally classified into two main groups, that is, pyramidal and nonpyramidal cells[4,6,11,17]. Pyramidal cells are projecting neurons with an apical dendrite and a long axon, while the majority of nonpyramidal cells are intrinsic neurons with no apical dendrite and a short axon[4,6]. Therefore, most of WGA labeled neurons in layers V/VI and layers II/III were regarded as pyramidal cells sending long projection fibers to thalamic MD. This coincided with the previous reports that the thalamic MD receives cortical connections chiefly from ACv and ACd[5,12]. However, WGA labeled neurons in deep layers of AId were regaraded as pleomorphic or stellate nonpyramidal cells with a long projecting axon[4].

Projections from anterior, laterodorsal and mediodorsal nuclei of the thalamus to the cingulate cortex have already been suggested in pre-

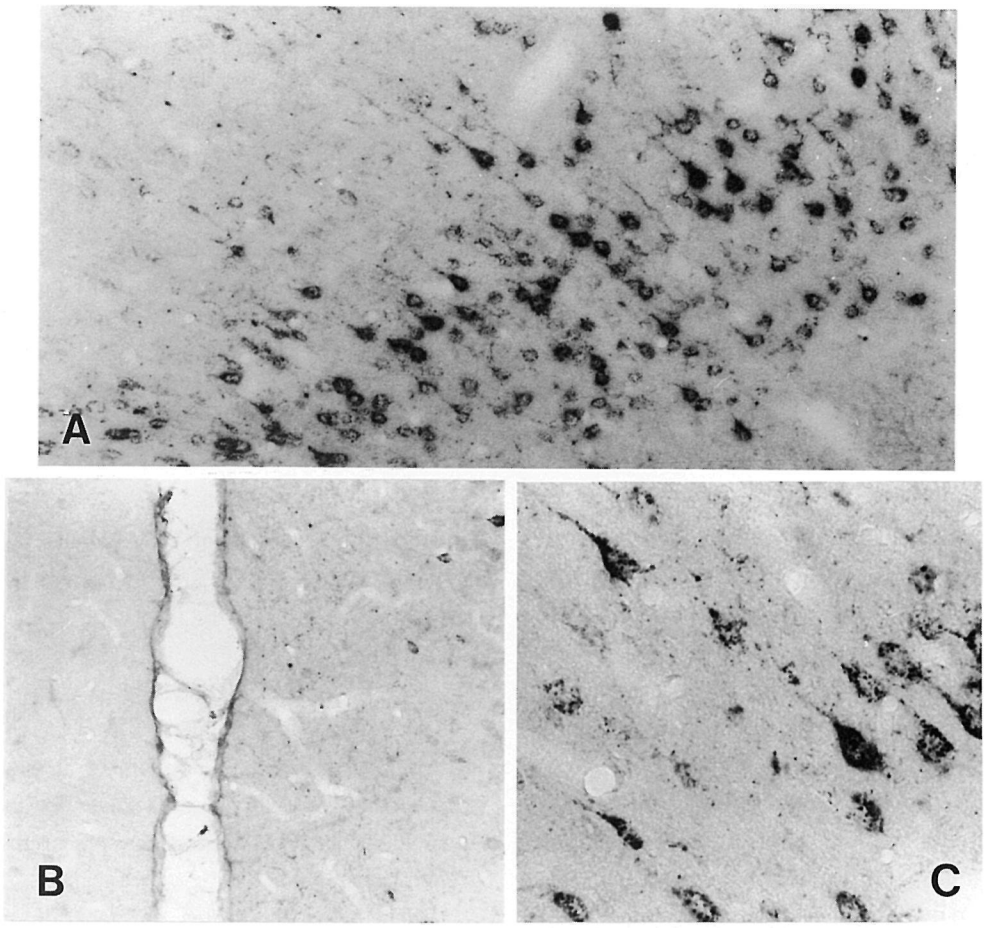

Fig. 2 A: WGA labeled neurons in layers V/VI of the medial mesocortex. ×240 B: Anterogradely labeled terminals appearing as grain-like profiles in the superficial layer on the ipsilateral side (right). ×240 C: Magnified WGA labeled neurons. ×380

vious experiments[7,8,11,16]. Reciprocal connections between layer VI and thalamic MD have been also reported[16]. Dense distribution of WGA grain profiles might imply the presence of numerous afferent fibers from thalamic MD in the superficial layer. In addition, the bilateral projection from both hemispheres to the thalamic MD became apparent because of the appearance of some WGA labeled neurons in contralateral mesocortex.

The cingulate cortex is a major component of the limbic system[15]. Thus, the fiber connections in the cingulate cortex might be seriously related to the function of limbic system[11,12,14,15]. Horikawa *et al.*[8] suggested that modulation of limbic system functions occurs via the direct thalamocortical connections. As mentioned before, the dense network of DA fibers derived from the mesencephalon might also contribute for the modulation of various convergent inputs to the anterior cingulate cortex[1,3]. Therefore, synaptology cancerning these afferent fibers and others from amygdala, septum, neostriatum, hypothalamus and brain stem nuclei, as well as intrinsic neurons ought to be investigated in detail. The further investigation is now undertaken by electron microscopical immunohistochemistry.

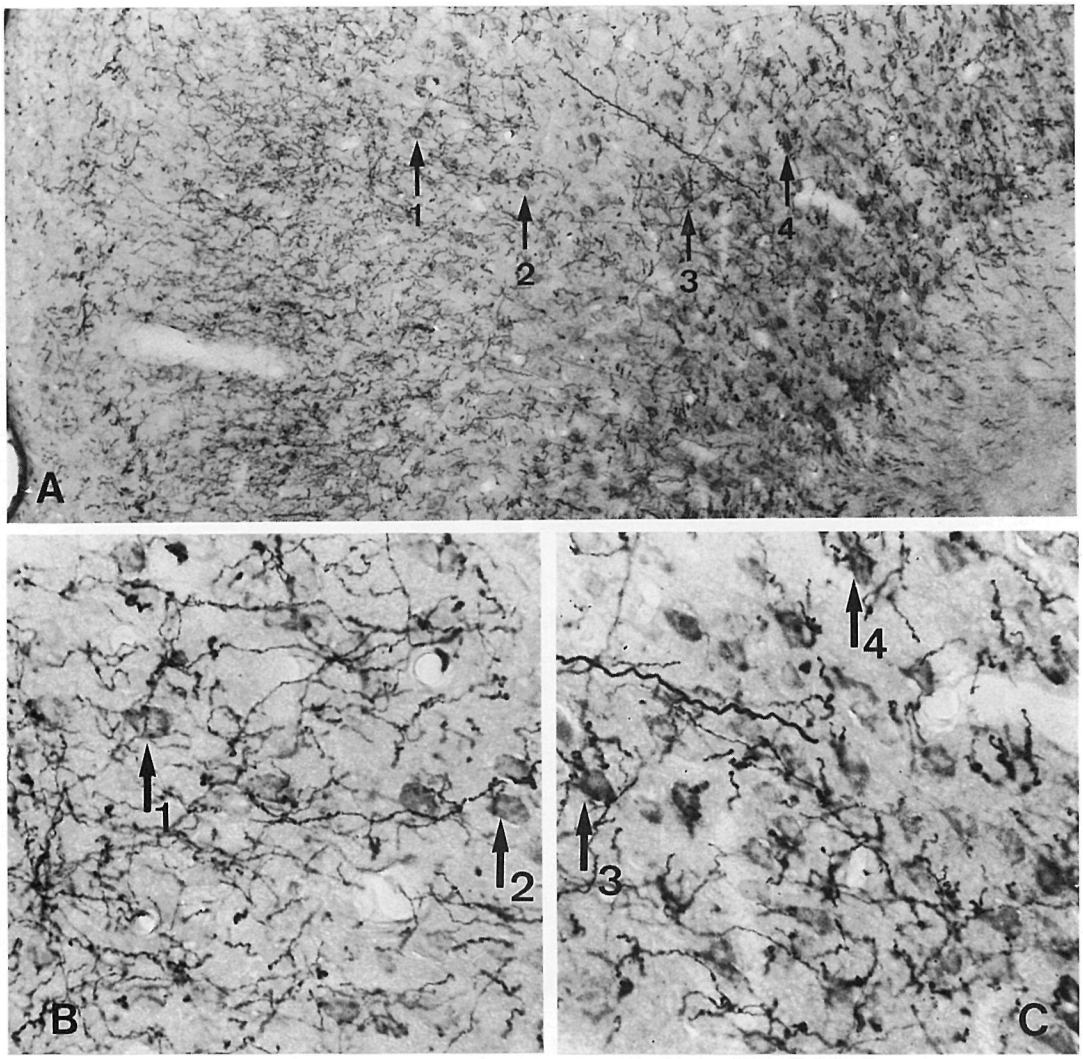

Fig. 3 A: WGA labeled neurons are stained blue and TH fibers are stained brown. Neurons indicated by arrows 1-4 correspond to those in B and C. ×130 B and C: ×380

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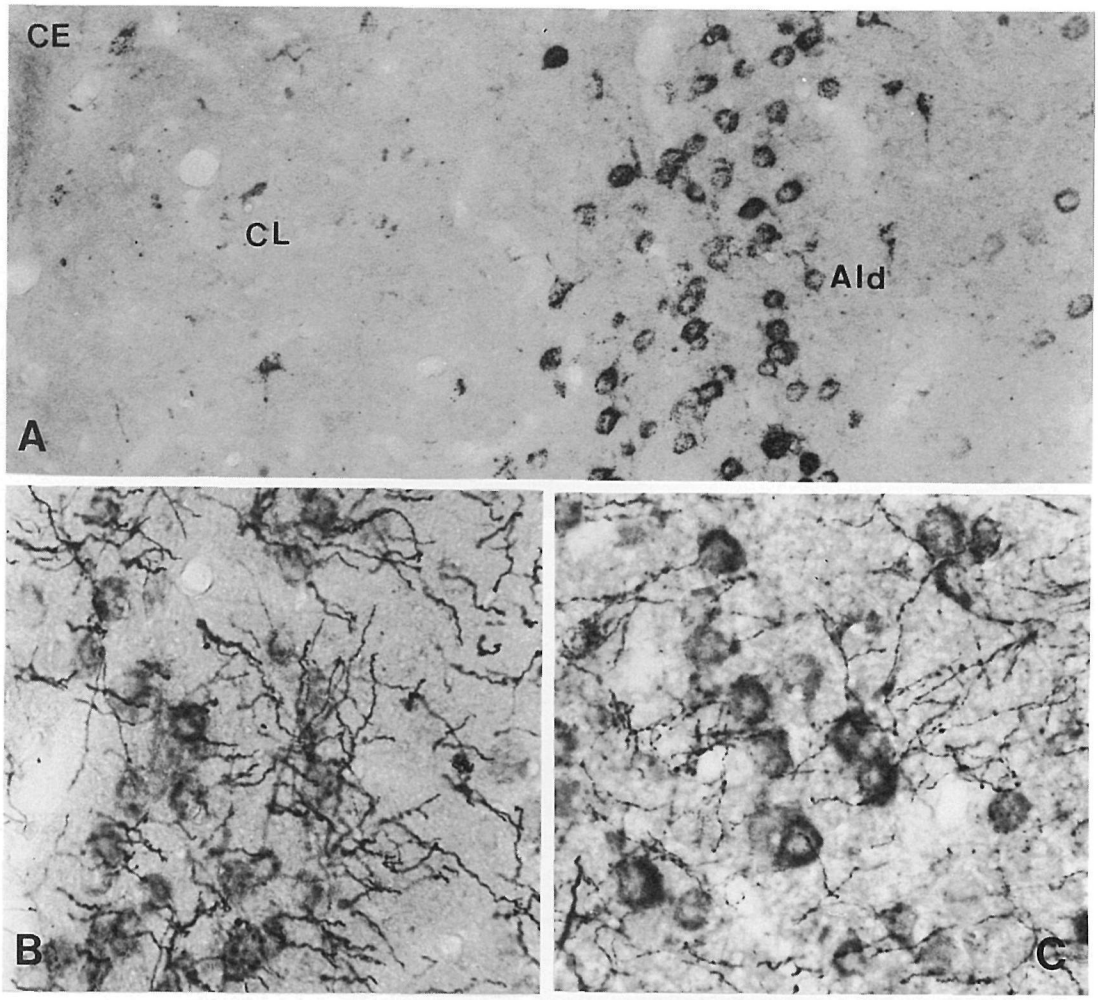

Fig. 4 A: WGA labeled neurons in AId *circumscribing the claustrum* (CL). CE: Capsula externa. ×260 B and C: WGA labeled neurons (blue) are surrounded by well-stained TH fibers (brown). ×380

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〈和文抄録〉

ラットの中間皮質における WGA 標識細胞と TH 免疫陽性線維の二重免疫染色

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一側の視床内側背側核（MD）に WGA を注入後，中間皮質の WGA 標識細胞と TH 免疫陽性線維を二重免疫染色法で観察した．同側では，WGA 標識錐体細胞は内側中間皮質のV/VI層とII/III層に相当数認められた．小数の WGA 標識細胞が運動野まで広がることはあったが，体性感覚野には全く見られなかった．外側中間皮質即ち前障を囲む部位では，標識細胞が小集団をなしていた．反対側の同部位でも常に標識細胞がみられたが，その数は同側の10%を越えなかった．WGA を順行性輸送した視床皮質投射線維は，同側の表層部に密な微細顆粒状で検出された．二重免疫染色では，青く染まった WGA 標識細胞が茶色の TH 免疫陽性瘤状線維終末で取り囲まれているのが観察された．